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Engineering an APRIL-specific B Cell Maturation Antigen*

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B cell maturation antigen (BCMA) is a tumor necrosis factor receptor family member whose physiological role remains unclear. BCMA has been implicated as a receptor for both a proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF), tumor necrosis factor ligands that bind to multiple tumor necrosis factor receptor and have been reported to play a role in autoimmune disease and cancer. The results presented herein provide a dual perspective analysis of BCMA binding to both APRIL and BAFF. First, we characterized the binding affinity of monomeric BCMA for its ligands; BAFF binding affinity (IC_{50} = 8 ± 5 μM) is about 1000-fold reduced compared with the high affinity interaction of APRIL (IC_{50} = 11 ± 3 nM). Second, shotgun alanine scanning of BCMA was used to map critical residues for either APRIL or BAFF binding. In addition to a previously described "DXL" motif (Gordon, N. C., Pan, B., Hymowitz, S. G., Yin, J., Kelley, R. F., Cochran, A. G., Yan, M., Dixit, V. M., Fairbrother, W. J., and Starovasnik, M. A. (2003) *Biochemistry* 42, 5977–5983), the alanine scanning results predicted four amino acid positions in BCMA (Tyr^{13}, Ile^{22}, Gln^{25}, and Arg^{27}) that could impart ligand specificity. Substitution of Tyr^{13} was tolerated for BAFF binding but not APRIL binding. Arg^{27} was required for high affinity binding to APRIL, whereas substitutions of this residue had minimal effect on affinity for BAFF. Further phage display experiments suggested the single mutations of I22K, Q25D, and R27Y as providing the greatest difference in APRIL *versus* BAFF binding affinity. Incorporation of the Q25D and R27Y substitutions into BCMA produced a dual specificity variant, since it has comparable binding affinity for both APRIL and BAFF, IC_{50} = 350 and 700 nM, respectively. Binding of the I22K mutant of monomeric BCMA to BAFF was undetectable (IC_{50} > 100 μM), but affinity for binding to APRIL was similar to wild-type BCMA. Based on these results, a BCMA-Fc fusion with the single I22K mutation was produced that binds APRIL, IC_{50} = 12 nM, and has no measurable affinity for BAFF. These results suggest that APRIL is the preferred ligand for BCMA and show that specificity can be further modified through amino acid substitutions.

The tumor necrosis factor receptors (TNFRs)[1] are a superfamily of transmembrane receptors involved in cell communication within the immune system. TNFR family members are structurally characterized by extracellular cysteine-rich domains (CRDs) that form ligand-binding motifs. Family members can be further classified based on intracellular domains that can either stimulate apoptosis through a death domain or cell proliferation through a TNF receptor-associated factor binding motif (1, 2). Downstream signaling for this subgroup of the TNFR superfamily activates the NF-κB intracellular pathway, often via TNF receptor-associated factors, ultimately resulting in cell proliferation (1–3). The corresponding TNF ligands share a common structural motif called the TNF homology domain, in the form of a β sheet jelly roll, through which the ligands trimerize for receptor activation. TNF family members are expressed in a membrane-bound form but can undergo proteolysis to produce an active soluble trimer.

Generally, members of the TNFR superfamily found on B or T cells are type I transmembrane proteins that have several CRDs (1). There are, however, receptors in a subgroup that are expressed by B cells, are type III transmembrane proteins, and contain a reduced number of CRDs: B cell maturation antigen (BCMA), transmembrane activator and CAML interactor (TACI), and BLyS (BAFF) receptor 3 (BR3, also called BAFF-R) (4–8). The extracellular domain (ECD) of TACI contains two CRDs, the BCMA ECD comprises one CRD, and the ECD of BR3 contains only a partial CRD. Together with the receptor (Fn14) for the TWEAK ligand, BCMA and BR3 are the smallest members of the TNFR superfamily. Since TACI, BCMA, and BR3 lack an intracellular death domain, it is believed that these receptors are involved in cell survival, proliferation, and/or differentiation. Specifically, recent *in vitro* studies of BCMA presented evidence for the BCMA intracellular region interacting with TNF receptor-associated factors, leading toward downstream activation of NF-κB, ELK-1, c-Jun, and p38 pathways (3). BCMA homozygous knockout mice, however, show no distinct phenotype, suggesting a redundant or nonessential role for this receptor (9, 10). In contrast, recent *in vivo* studies with TACI reveal its critical role in B cell homeostasis and mice harboring a knockout of the TACI gene develop a fatal, lupus-like autoimmune disease (11). A mouse strain expressing a BR3 receptor having intact extracellular and transmembrane domains but a disrupted intracellular domain, and thus defective for downstream signaling, has a significantly reduced number of mature peripheral B cells (7). Thus, BR3 appears to be essential for B cell survival, TACI is important for modulating the B cell population, and the physiological role of BCMA is unclear.

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[1] The abbreviations used are: TNFR, tumor necrosis factor receptor; CRD, cysteine-rich domain; TNF, tumor necrosis factor; BCMA, B cell maturation antigen; TACI, transmembrane activator and CAML interactor; BAFF, B cell-activating factor; BR3, BLyS (BAFF) receptor 3; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; NNS, (A/C/GT) (A/C/G/T) (G/C) codon.

The TNF family member BAFF is the only known ligand for BR3. BAFF-dependent B cell proliferation appears to require BR3; however, BAFF has also been reported to bind TACI and BCMA (6, 8, 12). APRIL, the TNF family member most closely related to BAFF, also binds TACI and BCMA (5, 7, 8). Despite cross-reactivity with receptors, the expression patterns of BAFF and APRIL are distinct; BAFF is expressed by macrophages, monocytes, neutrophils, dendritic cells, and radioresistant stromal cells, whereas APRIL is expressed by lymphoid cells and at elevated levels by some tumor cells (13–18). Tight regulation of BAFF levels appears to be critical for B cell homeostasis. BAFF knockout mice display significant reduction in the development and survival of follicular and marginal B cells, whereas mice expressing a BAFF transgene develop a lupus-like autoimmune syndrome (4, 9, 19, 20). Based on their roles in B cell-mediated immunity, these ligands (BAFF and APRIL) and their corresponding three receptors (BR3, BCMA, and TACI) provide likely targets for disease therapy. For example, studies by Kayagaki *et al.* (21) reveal attenuation of autoimmune lupus-like disease progression in mice with BR3-Fc treatment. In light of the receptor cross-reactivity, an APRIL-specific receptor would be a useful laboratory diagnostic tool or even a therapeutic agent; however, an APRIL-specific receptor has not yet been characterized.

Given that TNF ligands are trimeric and can bind three molecules of the corresponding receptor, there may be significant differences in apparent affinity between ligands binding to receptors in monovalent *versus* multivalent forms. Previous reports of binding affinities suggest that BAFF-BCMA has nanomolar affinity; however, these studies used a bivalent receptor-Fc fusion construct that could result in measured affinities that are enhanced by avidity (5, 6, 8). In this study, we characterized the APRIL and BAFF binding affinities of the extracellular domains of monomeric BCMA, BCMA-Fc, and BR3-Fc in order to determine the role that avidity could play in ligand-binding affinity. Furthermore, we have performed an alanine scan (22, 23) of the BCMA ECD in order to identify the residues determining specificity for binding to APRIL or BAFF. Finally, based on the alanine scan results, we produced a BCMA-Fc fusion with a single point mutation in the BCMA ECD, I22K, that maintains high affinity for APRIL but has no measurable binding to BAFF.

EXPERIMENTAL PROCEDURES

Reagents were obtained from the following sources: o-phenylenediamine dihydrochloride (Sigma), Streptavidin peroxidase (Roche Applied Science), IgG-horseradish peroxidase (Jackson ImmunoResearch Laboratories), Protease Complete (Roche Applied Science), anti-M13-horseradish peroxidase (Roche Applied Science), sulfo-*N*-hydroxysuccinimide-biotin (Pierce).

Human BAFF was expressed and purified as previously described (24). Human BR3-Fc (25) was a kind gift from Y.-M. Hsu (Biogen).

APRIL Expression and Purification—A PCR product coding for amino acids Lys^{104}–Leu^{241} of murine APRIL was subcloned into the XhoI/NotI sites of a modified pET-32a vector with a deleted S-Tag and enterokinase site to generate an N-terminal thioredoxin fusion protein. The pET-32a-APRIL(Lys^{104}–Leu^{241}) was transformed into Origami (DE3) competent cells (Novagen). Overnight cultures were diluted 1:100 and grown at room temperature in LB medium with 50 μg/ml carbenicillin to an A_{600} of 0.8 with vigorous shaking. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM for induction, and cultures were grown overnight at 25 °C. One liter of frozen cell pellet was resuspended in 100 ml of buffer A (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine) with 5 mM imidazole and placed on ice for 30 min. Cells were homogenized by passage through a microfluidizer and centrifuged at 15,000 rpm for 45 min. Supernatant was loaded onto a 3-ml nickel-nitrilotriacetic acid-agarose column (Qiagen), washed with 10 column volumes of Buffer A with 10 mM imidazole, and eluted with 10 column volumes of Buffer A with 300 mM imidazole. Fractions containing thioredoxin-APRIL fusion protein were pooled, concentrated, and purified by Superdex 200 size exclusion chromatography.

Baculovirus Expression and Purification of BCMA ECD—DNA coding for residues 4–53 of the human BCMA ECD was amplified by PCR and subcloned into the vector pET15b (Novagen) using the NdeI and XhoI restriction sites to introduce an N-terminal His tag and a thrombin cleavage sequence preceding the BCMA coding region. This His-tagged BCMA construct was subcloned into the baculovirus transfer vector pAcGP67B (Pharmingen) using the BamHI and NotI restriction sites. The transfer vector was co-transfected with BaculoGold DNA into Sf9 cells, and recombinant virus was subsequently isolated and amplified to facilitate protein production. After 3 days of growth of virally infected Hi5 cells at 27 °C, His-tagged protein was purified from the culture medium by chromatography on Ni^{2+}-nitrilotriacetic acid resin as described previously (26), followed by gel filtration on a Superdex 75 column. BCMA eluted from the Superdex 75 column as a monomer. N-terminal sequencing and mass spectrometry were used to confirm the proper identity of the purified protein and the presence of both glycosylated and nonglycosylated species in the purified protein pool, respectively.

BCMA-Fc Expression and Purification—DNA encoding the ECD (residues 5–51) of hBCMA was fused to hFc to form a fusion construct in the pRK vector and expressed in Chinese hamster ovary cells as previously described (5). Site-directed mutagenesis (Stratagene QuikChange™ method) was used to introduce the single mutant I22K in the hBCMA-hFc fusion construct. *Escherichia coli* 294 cells were transformed with the pRK-hBCMA-hFc plasmid for large scale production of plasmid DNA. Transient transfections of the plasmid in HEK 293 cells using FuGENE 6 (Roche Applied Science) produced secreted BCMA-Fc protein. The BCMA-Fc was purified from the HEK 293 growth medium by affinity chromatography using Protein A-Sepharose (Amersham Biosciences) resin.

Phage Display of BCMA—An initial vector for phage display of the BCMA extracellular domain was prepared by PCR subcloning of the fragment encoding residues 5–50 into the phagemid sTF-g3 (27). The resulting construct (BCMA1-g3) contained residues 5–50 fused at the C terminus to a tripeptide (G-S-A) linker and an amber stop codon followed by the C-terminal half of the M13 p3 coat protein. The bacterial signal sequence stII was joined to the N terminus of BCMA with an inserted Ser residue comprising the P1′ cleavage site for the signal peptidase. Expression was driven by the alkaline phosphatase promoter. Phagemid BCMA2-g3 was prepared by using site-directed mutagenesis (28) to insert the peptide epitope (MADPNRFRGKDLGG) for an antibody (3C8:2F4; Genentech, Inc.) between the P1 and P1′ residues of the signal sequence cleavage site. BCMA2-g3 phagemid was used to prepare two "shotgun alanine" scanning libraries essentially as described previously (29). A shotgun alanine codon will code for the wild-type residue, alanine, or one of two additional substitutions in certain cases, due to codon degeneracy, at a given position. Each of these libraries, prepared separately, contains shotgun codons at unique positions. Library 1 has shotgun codons at positions 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19; library 2 has shotgun codons at positions 22, 23, 25, 26, 27, 29, 30, 31, 32, 33, 34, 35, and 36. Each library contained at least 1×10^{11} phage/ml, allowing for complete representation of the theoretical diversity (>10^5-fold excess) (library 1 codes 1.1×10^6 unique sequences, and library 2 codes 5.2×10^5 unique sequences).

Library Sorting and Analysis—Phage from each of the libraries described above were subjected to rounds of binding selection against either BAFF, APRIL, or anti-tag antibody (3C8:2F4; Genentech, Inc.) immobilized on 96-well Nunc Maxisorp immunoplates (30). Bovine serum albumin-coated wells were used to determine nonspecific background binding. Phage eluted from each target were propagated in *E. coli* XL1-Blue in the presence of M13K07 helper phage; amplified phage were used for selection against the same target in the previous round. Phage sorting was stopped, generally at round 2 or 3, when 100-fold enrichment was obtained. Enrichment was calculated from the ratio of the phage titer eluted from the target-coated wells to the phage titer eluted from the bovine serum albumin-coated wells. Individual clones from each library and selection target were then grown in a 96-well format in 400 μl of 2YT medium supplemented with carbenicillin and KO7 helper phage. Phage ELISA assays (29) were performed to detect phage-displayed variants of BCMA ECD capable of binding BAFF, APRIL, or anti-tag antibody. All clones tested that were found to be positive in their respective ELISAs were then sequenced as described previously (30). Sequences of acceptable quality were translated and aligned. For selection of binding to BAFF, 40 and 47 sequences were analyzed for libraries 1 and 2, respectively. For selection of binding to APRIL, 44 and 46 sequences were analyzed from libraries 1 and 2,

respectively. For the display selection, binding to anti-tag antibody, a minimum of 40 sequences were analyzed for each library. To quantify the effect of each mutation on ligand binding, normalized frequency ratios (F) for each amino acid position were calculated from a ratio of ligand selection to display efficiency selection, as described previously (31).

NNS Library Construction and Sorting—The BCMA2-g3 phagemid was used to prepare a library having complete randomization (NNS degenerate codon as defined by IUB code (23)) of residues Leu^{16}, Ile^{22}, Gln^{25}, and Arg^{27}. The library contained 1×10^{10} phage/ml, allowing complete representation of the library, theoretically 1×10^{6} unique members. This library was sorted as described for the shotgun alanine libraries. Since each amino acid position selected for NNS codon introduction has the potential of all 20 amino acids from 31 triplet codons, the data are weighed according to codon degeneracy by calculating the ratio of percentage of occurrence to percentage of degeneracy of the amino acid at a given position as suggested previously (32). The normalized F' value is the percentage of occurrence to percentage of degeneracy ratio for ligand selection divided by the percentage of occurrence to percentage of degeneracy ratio for display efficiency.

Expression and Purification of BCMA-Z Fusion Proteins—Plasmid BCMA-Z, designed to express a protein fusion of BCMA ECD (residues 5–50) and the Z domain of protein A (33) was constructed by using PCR to replace the amber stop and gene 3 portion of BCMA1-g3 with the Z domain fragment from plasmid pZCT (34). Oligonucleotide-directed mutagenesis was performed as described (28) to generate point mutations and all constructs were verified by DNA sequencing. BCMA-Z fusion proteins were expressed by secretion from *E. coli* and purified by chromatography on IgG-Sepharose as described previously (35). BCMA-Z proteins were further purified by size exclusion chromatography on a HiPrep 16/60 Sephacryl S-100 HR column. BCMA-Z had an elution volume from the S-100 column between that of soluble human tissue factor (M_r 24,800) and *E. coli* thioredoxin (M_r 11,675). A molar mass of 12,000, consistent with the monomer MW of 13017 calculated from the amino acid sequence, was calculated from light scattering data collected on a mini-DAWN detector (Wyatt Technologies). Amino acid analysis was performed on the purified BCMA-Z to determine the extinction coefficient ($\epsilon_{280} = 9832\ M^{-1}\ cm^{-1}$).

Competitive Displacement ELISA—Receptors were tested for binding to either APRIL or BAFF in a competition ELISA assay. A 100-μl solution of carbonate buffer (pH 9.6) containing 2 μg/ml target ligand, either APRIL or BAFF, was coated on Nunc Maxisorp 96-well plates overnight at 4 °C. The plate was washed with PBS and blocked for 1 h with 200 μl of 0.2% bovine serum albumin in PBS. In one set of experiments, 0.2 μg/ml of BCMA-Z was added to 3-fold serial dilutions of ligand that were prepared in a 96-well plate containing PBS plus 0.05% Tween 20 and incubated for 1 h. After washing the coated plate with PBS plus Tween 20, 100 μl/well of each dilution was transferred and incubated for 1 h at room temperature. The plate was washed with PBS plus Tween 20 and incubated with 100 μl/well of a 1:3000 dilution of IgG-horseradish peroxidase for 1 h to detect bound BCMA-Z through the Z domain-IgG interaction. After washing the plate with PBS plus Tween 20 followed by a final wash in PBS, the plate was incubated for 5 min with 100 μl/well PBS substrate solution containing 0.8 mg/ml *o*-phenylenediamine dihydrochloride (Sigma) and 0.01% H_2O_2. The reaction was quenched with 100 μl/well of 1 M H_3PO_4, and the plate was read at 492 nm.

In a variation of the above ELISA, 3-fold serial dilutions of receptors were prepared in PBS plus 0.05% Tween 20 with 7 pM biotinylated BCMA-Z (when APRIL was the target) or 0.3 pM biotinylated "mini-BR3," BR3 residues 17–42 (when BAFF was the target). Mini-BR3 was prepared and biotinylated as described previously (24). For BCMA-Z biotinylation, 20 μg of purified BCMA-Z was incubated with a 3-fold molar excess of biotin-sulfo-*N*-hydroxysuccinimide (Pierce) in PBS at 25 °C for 3 h and then quenched with a 10-fold molar excess of Tris-HCl, pH 7.5. After washing the NUNC plate coated with either APRIL or BAFF with PBS plus Tween 20, 100 μl/well of each receptor dilution was transferred and incubated for 1 h at room temperature. The plate was washed with PBS plus Tween 20 and incubated with 100 μl/well of 0.1 unit/ml Streptavidin peroxidase (Roche Applied Science) for 15 min. The peroxidase signal was developed and detected as described above.

Measurement of Binding Constants for Receptors to Ligands—Surface plasmon resonance measurements on a BIAcore 3000 instrument (Pharmacia Biosensor) were used to measure binding affinities of receptors to immobilized APRIL and BAFF. The ligands were coupled to the sensor chip at a level of 400 resonance units using the amine coupling protocol supplied by the manufacturer. In all experiments, flow cell 1 was ethanolamine-blocked and used as the reference cell. Regeneration conditions that caused receptor dissociation, without disruption of the ligand trimers, could not be identified. Alternatively, in order to perform several binding measurements over the same flow cell, the ligand-receptor complex was allowed to dissociate by washing the flow cell with buffer at 30 μl/min for 900 s. Sensorgrams were recorded for receptor solutions ranging in concentration from 6.25 to 200 nM in 2-fold increments. Nonlinear regression analysis was used to simultaneously calculate kinetic constants and binding constants using software provided by the manufacturer.

RESULTS

BCMA Production—Previous reports on the interaction of BCMA with BAFF utilizing bivalent BCMA constructs (BCMA-Fc) have indicated a high affinity interaction with K_D values of about 1 nM (5, 8). More recently, studies with monovalent BCMA-Fc have indicated a much weaker affinity for BAFF (25). Here we have produced monomeric BCMA and measured affinities for both BAFF and APRIL. Monomeric BCMA was purified from baculovirus expression as well as from *E. coli* secretion as a fusion with the Z domain of Protein A. Although baculovirus expression provides a means to generate correctly folded and monomeric BCMA ECD, the Z domain fusion construct has the advantages of high level *E. coli* expression and detection through the Z-IgG interaction. BCMA-Z was readily purified from *E. coli* extracts by using a two-column procedure, and the purified protein eluted as a monomer from a gel filtration column (data not shown).

Measurement of Receptor-Ligand Binding Affinities—Binding affinity for monomeric human BCMA-Z to murine APRIL and human BAFF was measured by the competition ELISA described under "Experimental Procedures" (Fig. 1*A*). Although human BCMA and human BAFF were used in these experiments, murine APRIL was used, since the murine protein is better behaved than the human protein *in vitro*. Murine and human APRIL share >80% sequence identity, and the putative receptor-binding residues (see "Discussion") are absolutely conserved. APRIL was expressed and purified as a thioredoxin fusion protein, and all binding experiments were done with the intact fusion protein, since removal of the thioredoxin by limited proteolysis resulted in reduced solubility. APRIL in solution was able to compete for binding to BCMA-Z with immobilized APRIL with an IC_{50} of 20 nM, whereas BAFF in solution competed for BCMA-Z binding with immobilized BAFF with an IC_{50} of >65 μM. Comparison of APRIL binding affinity for BCMA-Z and BCMA from baculovirus expression in a competition ELISA shows that the Z domain does not influence the measured affinity (Fig. 1*B*). This equivalent binding allowed the remaining BCMA binding measurements to be made using BCMA-Z. A competition ELISA measuring BCMA-Z binding to APRIL and BAFF using biotinylated BCMA-Z (for APRIL binding) or biotinylated mini-BR3 (24) (for BAFF binding) confirms that the affinity measurements of BCMA-Z binding to receptors (Fig. 1*C*) are consistent with the ligand competition data; IC_{50} values of BCMA-Z binding to APRIL (IC_{50} = 11 nM) are about 1000-fold higher in affinity than BCMA-Z binding to BAFF (IC_{50} = 8 μM for BAFF).

Since previously reported binding affinities of BCMA-BAFF interaction have been reported to be in the low nanomolar range (5, 8, 25), a comparison of various receptor constructs and ligands was done to examine the effects of avidity on receptor binding to APRIL and BAFF. Fig. 2 compares binding affinities of BCMA-Z, BCMA-Fc, and BR3-Fc to either BAFF (Fig. 2*A*) or APRIL (Fig. 2*B*). These data reveal that the monovalent BCMA-Z binds to BAFF (IC_{50} = 9 μM) with a 1000-fold reduced affinity compared with APRIL (IC_{50} = 7 nM), whereas BCMA-Fc, a bivalent construct, binds to both APRIL and BAFF with low nanomolar affinity (IC_{50} = 5 and 13 nM, respectively).

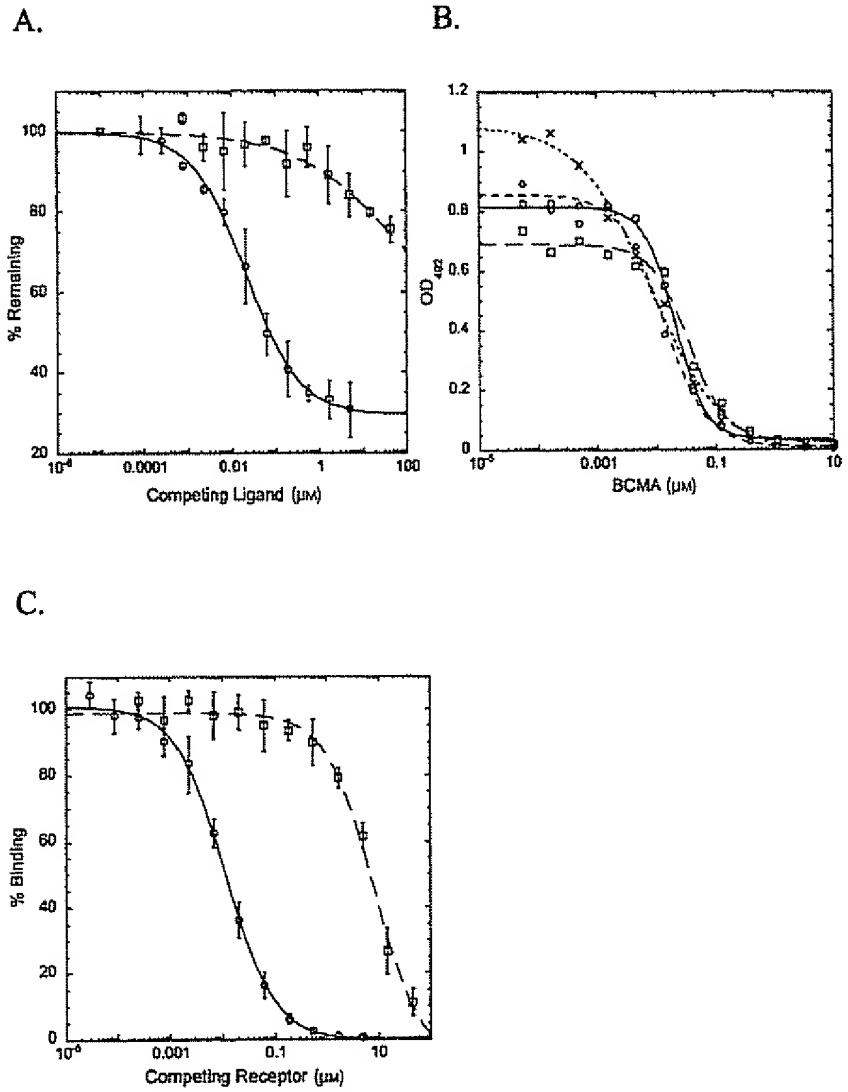

FIG. 1. **Competitive displacement ELISA of BCMA ligand interaction.** *A*, inhibition of BCMA-Z binding to immobilized APRIL (*circles*) or BAFF (*squares*) in the presence of increasing amounts of the same soluble ligand. Data represent the mean ± S.D. of three data sets with IC_{50} values of 20 nM for APRIL and >65 μM for BAFF. *B*, displacement of biotinylated BCMA binding to immobilized APRIL by unbiotinylated BCMA (*circles*, biotinylated baculovirus BCMA and unlabeled baculovirus BCMA; *squares*, biotinylated baculovirus BCMA and unlabeled BCMA-Z, *diamonds*, biotinylated BCMA-Z and unlabeled baculovirus BCMA, ×, biotinylated BCMA-Z and unlabeled BCMA-Z). *C*, data shown are for BCMA-Z competing with biotinylated BCMA-Z for binding to immobilized APRIL (*circles*; IC_{50} = 11 nM) or BCMA-Z competing with biotinylated BR3 for binding to immobilized BAFF (*squares*; IC_{50} = 8 μM). Data represent the mean ± S.D. of three data sets, and *curves* represent fitting to a four-parameter equation.

BR3-Fc binds BAFF, its predicted physiological ligand, with IC_{50} = 9 nM for murine BR3-Fc and IC_{50} = 7 nM for human BR3-Fc, but neither human nor murine BR3-Fc have measurable affinity for APRIL.

The ligand binding affinity of BCMA was also measured by surface plasmon resonance. APRIL and BAFF were selected for immobilization due to nonspecific interactions of APRIL with the sensor chip surface when used in the mobile phase. Table I summarizes the kinetics data for BCMA-Z and BCMA-Fc. BCMA-Z bound to immobilized APRIL with a K_D value of 5.5 nM, whereas binding to BAFF was not detectable. BAFF binding by BCMA ECD produced via baculovirus expression was also undetectable. In contrast, BCMA-Fc bound to both BAFF (K_D = 4.9 nM) and APRIL (K_D = 0.2 nM) with high affinity, consistent with the competition ELISA result. BCMA-Fc binding to immobilized APRIL gave a faster on-rate with a slower off-rate than measured for the BCMA-Z-APRIL interaction, as expected for a bivalent molecule where avidity contributes to binding.

Mutational Analysis of BCMA—A shotgun alanine scan (29) of the single extracellular CRD of BCMA was used to determine the contribution of individual amino acid side chains to the binding of either APRIL or BAFF. In order to generate phage libraries that were completely represented by the phage pool,

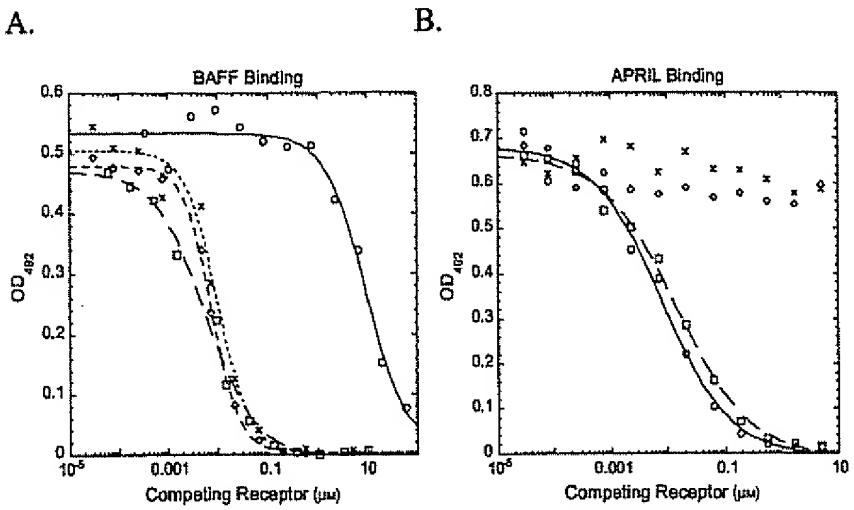

FIG. 2. **Comparison of monovalent and bivalent receptors for binding to immobilized ligand.** *A*, displacement of biotinylated mini-BR3 binding to immobilized BAFF by BCMA-Z (*circles*), BCMA-Fc (*squares*), human BR3-Fc (*diamonds*), or murine BR3-Fc (×). *B*, inhibition of biotinylated BCMA-Z binding to immobilized APRIL by BCMA-Z (*circles*), BCMA-Fc (*squares*), human BR3-Fc (*diamonds*), or murine BR3-Fc (×).

TABLE I

Binding constants for receptor binding to ligands

Association (k_a) and dissociation (k_d) rate constants and dissociation constants (K_D) were calculated by nonlinear regression analysis using a 1:1 binding model. NMB, no measurable binding.

Receptor-Ligand	k_a	k_d	K_D
	$\times 10^{-5}\ M^{-1}\ s^{-1}$	$\times 10^{-1}\ s^{-1}$	nM
BCMA-Fc-APRIL	31.2	0.1	0.2
BCMA-Z-APRIL	8.5	4.7	5.5
BCMA-Fc-BAFF	6.8	3.4	4.9
BCMA-Z-BAFF	NMB	NMB	NMB
BCMA(baculovirus)-BAFF	NMB	NMB	NMB

the ECD was divided into two distinct libraries. In these libraries, wild-type codons were replaced by degenerate codons at amino acid positions 7–36 in the BCMA ECD (except cysteines and alanines), resulting in either the wild-type amino acid or alanine being expressed at the selected sites. For positions where the wild-type residue is Arg, Asn, Gln, His, Ile, Leu, Phe, or Tyr, the shotgun code allows for two additional amino acid substitutions. These sites were chosen for mutagenesis on the basis of their proximity to BAFF in the BCMA-BAFF crystal structure (36) and because of their structural equivalence to residues found important for BAFF-binding in a shotgun scan of BR3 (24). The BCMA phage libraries were individually subjected to two types of selection, target ligand selection (BAFF or APRIL) and display selection, by binding to an antibody that recognizes an epitope tag N-terminally displayed on all BCMA library members (31). Display selection is critical for normalizing BAFF- and APRIL-binding selection with respect to expression differences between libraries. The normalized wild type/alanine ratios (F) obtained at each position for both BAFF and APRIL selection are shown in Fig. 3.

The F value of an amino acid describes the effect of substitution on target binding relative to display efficiencies. F values of >1 represent deleterious mutations, whereas values of <1 represent favorable mutations. Due to the relatively small pool of enriched sequences analyzed, only F values representing a greater than 10-fold effect are considered significant. The Tyr^{13} position in BCMA tolerated some alanine, as well as aspartate and serine, substitution for BAFF-binding but was absolutely conserved for APRIL-binding. The F values for Phe^{14} indicate a significant contribution to BAFF binding but a more modest effect on affinity for APRIL. For both BAFF and APRIL binding, amino acids Asp^{15} and Leu^{17} are absolutely critical in that only wild-type residues were selected. A conservative substitution of Leu^{18} to Val was observed for both APRIL and BAFF selection; however, alanine was not observed at this position. Ala substitution of Ile^{22} was not tolerated for binding to BAFF but was compatible with APRIL binding. The Gln^{25} to Ala replacement appeared to have opposite effects on APRIL and BAFF binding, but the difference in F value was barely significant. In contrast, Ala substitution of Arg^{27} was strongly preferred for binding to BAFF and disfavored for APRIL binding. Other replacements in the C-terminal portion of BCMA had no effect on ligand binding.

Mutagenesis results for residues Ile^{22}, Gln^{25}, and Arg^{27} suggest these positions as likely candidates for providing ligand specificity, since Ala substitution had opposite effects on BAFF and APRIL binding. Positions Leu^{18}, Ile^{22}, Gln^{25}, and Arg^{27} were selected for further phage optimization studies by incorporation of NNS degenerate codons at these positions followed by selection for ligand binding. This new phage library was subjected to three rounds of sorting against either BAFF or APRIL and compared with the display target antibody. A large F' value for an amino acid at a given position indicates that that amino acid is a favorable substitution for binding to the target ligand. As shown in Table II, the substitutions that result in the maximum difference in F' (23, 32) between APRIL binding and BAFF binding are I22K, Q25D, and R27Y. The I22K substitution produced a >12-fold preference for APRIL binding over BAFF binding; Q25D and R27Y resulted in >13- and 9.5-fold preference for BAFF binding relative to APRIL binding, respectively.

In order to confirm the effects on ligand binding indicated by the phage display results, point mutants were produced as BCMA-Z fusion proteins, purified, and assayed for BAFF and APRIL binding by competitive displacement ELISA (Table III). These data show that Tyr^{13} is critical for BCMA binding to APRIL, since Ala, Ser, or Phe substitutions of this residue all increased the IC_{50} by at least 400-fold. The substitution of Tyr^{13} with Ala, Ser, or Phe, produced only small changes in affinity for BAFF. Consistent with the phage display results,

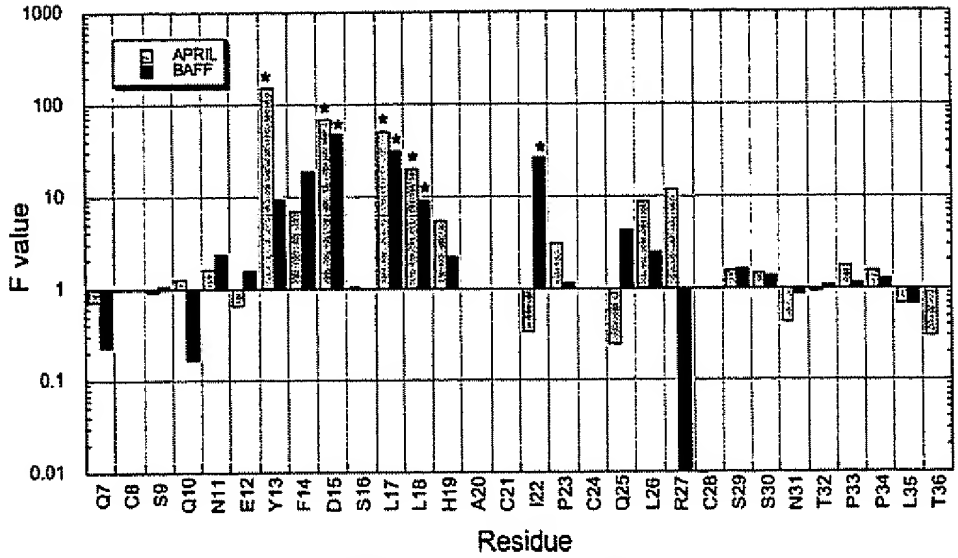

FIG. 3. **Shotgun alanine scan mutagenesis of BCMA for binding to BAFF or APRIL.** The normalized frequency ratios (*F*) observed for each of the scanned positions in BCMA obtained from sequences of positive clones after two rounds of selection for binding to BAFF (*solid*) or APRIL (*hatched*). *F* values were calculated as described under "Experimental Procedures." Those *bars* with an *asterisk above* indicate values that represent a lower limit, since Ala was not observed at these positions.

TABLE II

Residue preferences at BCMA positions 18, 22, 25, and 27 for binding to BAFF or APRIL

A library having complete randomization (NNS codons) at these four sites was prepared and sorted for binding to BAFF or APRIL. The normalized *F* values for each of the scanned positions in BCMA were obtained from sequences of positive clones after three rounds of selection for binding to BAFF or APRIL. Normalized frequency values (*F'*) are calculated from the target/display ratio taking into consideration codon degeneracy as described under "Experimental Procedures." In boldface are the *F* values that represent a greater than 10-fold change in frequency. A zero value indicates a position where the amino acid was not observed in screened clones.

Amino acid	*F* BAFF				*F* APRIL			
	Leu^{18}	Ile^{22}	Gln^{25}	Arg^{27}	Leu^{18}	Ile^{22}	Gln^{25}	Arg^{27}
Ala	0	6	0	1	0	0	0	0
Cys	0	0	0	0	0	0	0	0
Asp	0	0	**13**	0	0	0	0	0
Glu	0	0	1	0	0	0	1	0
Phe	0	0	0	1	0	0	0	0
Gly	0	0	2	0	0	0	3	0
His	0	0	0	5	0	0	0	0
Ile	**18**	6	9	5	8	6	0	0
Lys	0	0	1	0	0	**12**	1	0
Leu	4	1	0	1	9	0	1	0
Met	0	0	0	1	0	6	6	1
Asn	0	0	0	0	0	0	6	0
Pro	3	0	0	0	0	0	3	0
Gln	0	0	9	0	0	0	**12**	0
Arg	0	1	2	0	0	0	4	**53**
Ser	0	0	1	4	0	1	4	0
Thr	6	0	2	1	0	1	0	0
Val	5	7	1	1	2	6	0	1
Trp	0	0	0	5	0	0	0	0
Tyr	0	0	9	**19**	0	0	6	2

the single amino acid substitution of I22K in BCMA-Z caused a greater than 10-fold reduction in affinity for BAFF but only about a 3-fold penalty for APRIL binding. The single substitution of Q25D in BCMA-Z did not significantly improve binding to BAFF relative to APRIL, whereas the single amino acid substitution of R27Y reduced APRIL binding 40-fold, compared with wild type. The double mutant Q25D/R27Y produced a receptor that could bind both APRIL and BAFF with nearly the same affinity. These substitutions gave nonadditive contributions to binding, since the effect measured for the double mutant was greater than the sum of effects measured for the single mutants. For example, the Q25D substitution resulted in decreased affinity for BAFF, but when combined with R27Y it gave an increased affinity.

Surface plasmon resonance was used to independently measure the binding constants of the mutant BCMA-Z proteins for APRIL (Table IV). Consistent with ELISA IC_{50} measurements, BCMA-Z wild-type, I22K, and Q25D all bound APRIL in the low nanomolar range. R27Y BCMA-Z binding to APRIL showed a 1000-fold reduction in affinity. The decreased affinity of this mutant results primarily from a decrease in the on rate of binding, which leads to poor fitting to a 1:1 binding model and probably underestimates the actual K_D. The surface plasmon resonance data for BCMA-Z Q25D/R27Y binding to APRIL was

TABLE III
Competitive displacement assay of BCMA-Z mutants binding to APRIL or BAFF

IC_{50} values shown are for BCMA-Z wild type (mean ± S.D. of four data sets) or BCMA-Z point mutants competing with biotinylated BCMA-Z for binding to immobilized APRIL or competition with biotinylated BR3 for binding to BAFF.

BCMA-Z	IC_{50} values	
	BAFF	APRIL
	μM	nM
Wild type	8 ± 5	11 ± 3
Y13A	12	5100
Y13S	6	8400
Y13F	3	5700
I22K	>100	38
Q25D	36	32
R27Y	4	400
Q25D/R27Y	0.7	350

TABLE IV
BCMA-Z binding to immobilized APRIL by surface plasmon resonance

The rate constants and dissociation constants (K_D) were calculated by nonlinear regression analysis using a 1:1 binding model.

BCMA-Z	k_a	k_d	K_D
	$\times 10^{-5}$ M^{-1} s^{-1}	$\times 10^{3}$ s^{-1}	nM
Wild type	5.5	4.7	5.5
I22K	11.0	6.9	6.3
Q25D	7.9	5.9	7.5
R27Y	0.1	25	6500

not well described by a 1:1 binding model such that the rate and equilibrium constants could not be calculated.

Measurement of BCMA-Fc Wild Type and I22K Binding to Ligands—Since the single point mutation I22K resulted in a BCMA-Z protein that maintained high affinity for APRIL and no measurable binding to BAFF, this mutation was introduced into BCMA-Fc protein to determine whether the affinity and specificity would be present in a bivalent format. The competition ELISA method, as described under "Experimental Procedures," was used to measure binding of BCMA-Fc wild type and I22K fusion proteins to APRIL or BAFF using biotinylated BCMA-Z for APRIL binding or biotinylated BR3 (24) for BAFF binding (Fig. 4). These data reveal that the single point mutation, I22K, in BCMA resulted in a bivalent protein that binds APRIL (IC_{50} = 12 nM) with similar affinity to the wild type BCMA-Fc (IC_{50} = 9 nM) but has no measurable affinity to BAFF (IC_{50} > 10 μM).

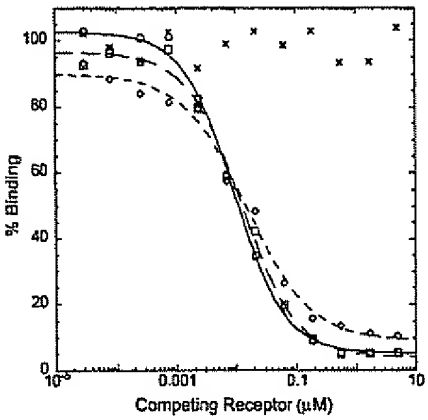

FIG. 4. **Competitive displacement ELISA of BCMA-Fc wild type and I22K mutant for ligand binding.** BCMA-Fc wild type (*circles*) and I22K mutant (*squares*) competing with biotinylated BCMA-Z for binding to immobilized APRIL and BCMA-Fc wild type (*diamond*) and I22K mutant (×) competing with biotinylated BR3 for binding to immobilized BAFF.

DISCUSSION

Monovalent BCMA binds APRIL with high affinity. In contrast, the affinity of BCMA for BAFF is 1000-fold weaker than for APRIL. Since the BCMA produced in *E. coli* binds APRIL with high affinity and is equivalent to material produced by expression in insect cells, the weak affinity for BAFF cannot be explained by a misfolding of BCMA-Z. A recent report also described the weak affinity of the monovalent BCMA-BAFF interaction (25); however, ours is the first report of a high affinity interaction between monovalent BCMA and APRIL. Consistent with earlier studies (5, 8, 25), the bivalent protein (BCMA-Fc) binds BAFF with an apparent high affinity. An avidity component originating from a bivalent receptor interacting with a ligand having three binding sites can enhance the apparent affinity. Thus, it is likely that BAFF may bind with high affinity to cells expressing BCMA only if the receptors are preorganized on the cell surface. Although it has been proposed that FAS and TNFR1 can form homo-oligomers on the cell surface in the absence of ligand (37), BCMA appears to lack the PLAD domain necessary for this association. Under normal physiological conditions, BCMA may operate only as a receptor for APRIL and not for BAFF. This conclusion is consistent with recent findings showing that the effects of BAFF on the development of B cells are independent of BCMA (9).

Similar to BR3, a DXL motif is presented at the tip of a type I β-turn in BCMA (21, 36). Shotgun alanine scanning of BCMA confirms that the DXL motif is critical for APRIL binding as well as BAFF binding (Fig. 5*A*). These results suggest that this portion of BCMA is bound in a cavity on APRIL that is similar to the pocket on BAFF for binding BCMA and BR3 (38–40). In the structures determined for the BR3-BAFF or BCMA-BAFF (Fig. 5*B*) complexes, the leucine residue of the DXL motif (Leu^{28} in BR3, Leu^{17} in BCMA) interacts with BAFF residues Ala^{207}, Leu^{211}, Ile^{233}, and Pro^{264}, with Gly^{209} forming the bottom of the pocket. The Asp residue of the DXL motif (Asp^{26} in BR3, Asp^{15} in BCMA) makes a salt bridge with BAFF residue Arg^{265}, and the conformation of the Asp may be stabilized through a hydrogen bond with BAFF residue Tyr^{206}. High resolution structures for APRIL by itself or in complex with BCMA are not available; however, APRIL is expected to have a similar binding pocket for the leucine side chain, since, as shown in Fig. 5*B*, the key residues are conserved (Gly^{209}, Ile^{233}, and Pro^{264}) or conservatively substituted (A207T, L211V). Arg^{265} is conserved in APRIL, thus allowing formation of a salt bridge with Asp^{15}.

Residues outside of the DXL motif would appear to confer the specificity of BCMA for APRIL and BR3 for BAFF. For example, mutation of Tyr^{13} has little effect on BAFF-binding (Table III), but even the subtle substitution of Tyr^{13} for phenylalanine results in a remarkable 520-fold decrease in APRIL binding affinity. The BAFF-BCMA crystal structure (36) shows that the hydroxyl group of Tyr^{13} has the potential to form hydrogen bonds with Asp^{15} and Arg^{27} on BCMA and/or Tyr^{206} on BAFF (Fig. 5). Our results indicate that none of these potential hydrogen bonds are important for BAFF binding. In contrast, the hydroxyl group is clearly essential for high affinity APRIL binding. Interestingly, the residue corresponding to Tyr^{206} in APRIL is a phenylalanine, suggesting that the importance of Tyr^{13} probably results from an intramolecular hydrogen bond within BCMA. Given the key role of Asp^{15} in the interface, the importance of Tyr^{13} to APRIL binding may be due to an indirect effect of influencing the position of Asp^{15}; in BAFF, such a role could be accomplished by Tyr^{206} on the ligand.

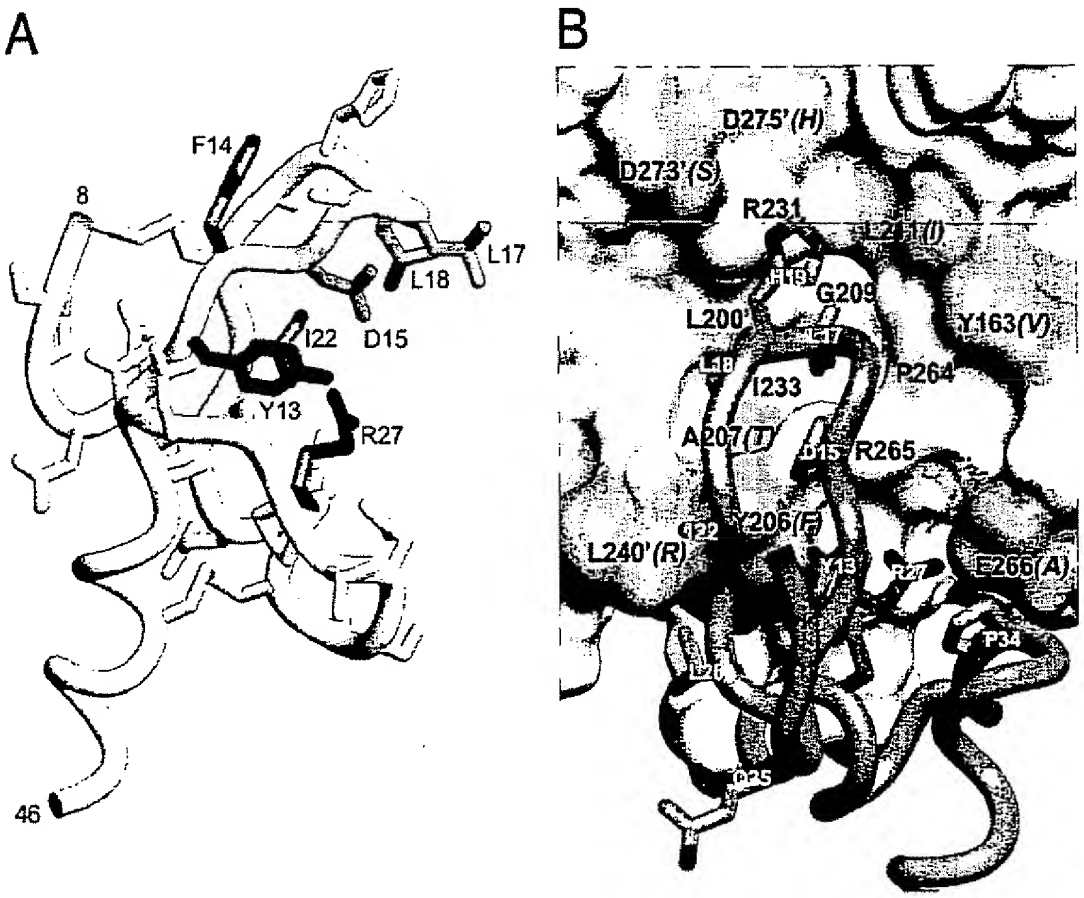

FIG. 5. **Functional and structural epitopes for ligand binding by BCMA.** *A*, summary of BAFF and APRIL binding determinants mapped onto the structure of BCMA (Protein Data Bank code 1OQD (36)). All side chains evaluated in the shotgun alanine scan are shown. Side chains *colored green* show a >10-fold decrease on binding to BAFF when substituted by alanine; those *colored blue* show >10-fold decrease on binding to APRIL when substituted by alanine; those *colored red* show >10-fold decrease on binding to both BAFF and APRIL when substituted by alanine. *B*, close-up of the BAFF-BCMA contact region from the ligand-receptor co-crystal structure (Protein Data Bank code 1OQD (36)); BAFF contact residues are labeled in *black type*, whereas those for BCMA are labeled in *white type*. BCMA is shown in a *blue ribbon*; only side chains that contact BAFF are shown, with the addition of Gln^{25}. BAFF is shown in a surface representation, with the portion of the surface that contacts BCMA *colored yellow* and *red*; *yellow* indicates residues that are identical between BAFF and APRIL; *red* indicates residues that differ between BAFF and APRIL, with the APRIL residue type shown *parenthetically* using single-letter amino acid code.

Arg^{27} of BCMA is also important for specifying APRIL *versus* BAFF-binding. Again, mutation of this position has little effect on BAFF binding but disrupts APRIL binding significantly (Tables III and IV). In the BAFF-BCMA crystal structure, Arg^{27} forms a salt bridge with Glu^{266}, yet the R27Y mutant indicates that this salt bridge is not required for BAFF association. This conclusion is consistent with the observation of a high affinity BAFF-BR3 interaction, since BR3 has a leucine (Leu^{38}) in the position analogous to Arg^{27}. Since APRIL has Glu^{266} replaced with Ala, it is unclear why Arg^{27} of BCMA is required for high affinity binding to APRIL. Further understanding of this effect will require determination of the BCMA-APRIL complex structure. In addition to the contributions from Leu^{38} interactions, the specificity of BR3 for BAFF over APRIL could be explained by the substitution of a cysteine residue (Cys^{24}, which makes a disulfide bond with Cys^{15}) for the residue equivalent to Tyr^{13}. Indeed, the C24Y mutation in BR3 increases affinity for APRIL (36), and hydrophobic residues at position 38 in BR3 are important for BAFF binding (24).

In addition to BCMA residues Tyr^{13} and Arg^{27}, our results from phage display experiments suggest that Ile^{22} and Gln^{25} are important for determining ligand specificity. Ile^{22} makes a hydrophobic contact with BAFF, and thus the I22K substitution results in a weaker affinity for BAFF. Interestingly, wild-type murine BCMA has Lys at position 22 and would likely have a weak affinity for mBAFF. The contact residues for Ile^{22} on BAFF, Tyr^{206}, and Leu^{240}, are replaced with Phe and Arg in APRIL. Given the positive charge on APRIL residue Arg^{240}, it is surprising that the I22K substitution has no effect on affinity for APRIL. The side chain of Gln^{25} is not in contact with BAFF, and thus the Q25D single mutation has no significant effect on APRIL or BAFF binding. However, Q25D did increase affinity for BAFF when combined with R27Y such that a dual specificity BCMA variant was obtained. Given that Arg^{27} and Gln^{25} point in opposite directions, the origin of this nonadditive effect is unclear.

Both BAFF and APRIL have been implicated in autoimmune diseases and carcinomas. APRIL expression has been shown to be up-regulated in many tumors including colon and prostate cancers (13, 41, 42). The BAFF-specific antagonist BR3-Fc (21),

together with studies on BAFF knockout mice (9), has been used to demonstrate the essential role of BAFF in B cell development. The role of APRIL in B cell homeostasis is unclear, especially since APRIL knockout mice display normal B cell levels (43). Soluble BCMA-Fc has been used to suggest a role for APRIL in humoral immunity (8) and tumor growth (41); however, since bivalent BCMA binds BAFF with high affinity, the observed effects could be a consequence of BAFF inhibition. These *in vivo* experiments could provide more conclusive evidence for an involvement of APRIL if monovalent BCMA was used as an antagonist. Additionally, the BCMA-Fc mutant I22K retains both high affinity and specificity for APRIL and should be useful for evaluating the role of APRIL in normal and disease states.

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REFERENCES

1. Bodmer, J. L., Schneider, P., and Tschopp, J. (2002) *Trends Biochem. Sci.* 27, 19–26
2. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) *Cell* 104, 487–501
3. Hatzoglou, A., Roussel, J., Bourgeade, M. F., Rogier, E., Madry, C., Inoue, J., Devergne, O., and Tsapis, A. (2000) *J. Immunol.* 165, 1322–1330
4. Gross, J. A., Johnston, J., Mudri, S., Enselman, R., Dillon, S. R., Madden, K., Xu, W., Parrish-Novak, J., Foster, D., Lofton-Day, C., Moore, M., Littau, A., Grossman, A., Haugen, H., Foley, K., Blumberg, H., Harrison, K., Kindsvogel, W., and Clegg, C. H. (2000) *Nature* 404, 995–999
5. Marsters, S. A., Yan, M., Pitti, R. M., Haas, P. E., Dixit, V. M., and Ashkenazi, A. (2000) *Curr. Biol.* 10, 785–788
6. Shu, H. B., and Johnson, H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 9156–9161
7. Thompson, J. S., Bixler, S. A., Qian, F., Vora, K., Scott, M. L., Cachero, T. G., Hession, C., Schneider, P., Sizing, I. D., Mullen, C., Strauch, K., Zafari, M., Benjamin, C. D., Tschopp, J., Browning, J. L., and Ambrose, C. (2001) *Science* 293, 2108–2111
8. Yu, G., Boone, T., Delaney, J., Hawkins, N., Kelley, M., Ramakrishnan, M., McCabe, S., Qiu, W. R., Kornuc, M., Xia, X. Z., Guo, J., Stolina, M., Boyle, W. J., Sarosi, I., Hsu, H., Senaldi, G., and Theill, L. E. (2000) *Nat. Immunol.* 1, 252–256
9. Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., Frew, E., and Scott, M. L. (2001) *Science* 293, 2111–2114
10. Xu, S., and Lam, K. P. (2001) *Mol. Cell. Biol.* 21, 4067–4074
11. Seshasayee, D., Valdez, P., Yan, M., Dixit, V. M., Tumas, D., and Grewal, I. S. (2003) *Immunity* 18, 279–288
12. Wu, Y., Bressette, D., Carrell, J. A., Kaufman, T., Feng, P., Taylor, K., Gan, Y., Cho, Y. H., Garcia, A. D., Gollatz, E., Dimke, D., LaFleur, D., Migone, T. S., Nardelli, B., Wei, P., Ruben, S. M., Ullrich, S. J., Olsen, H. S., Kanakaraj, P., Moore, P. A., and Baker, K. P. (2000) *J. Biol. Chem.* 275, 35478–35483
13. Hahne, M., Kataoka, T., Schroter, M., Hofmann, K., Irmler, M., Bodmer, J. L., Schneider, P., Bornand, T., Holler, N., French, L. E., Sordat, B., Rimoldi, D., and Tschopp, J. (1998) *J. Exp. Med.* 188, 1185–1190
14. Moore, P. A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D. W., Feng, P., Soppet, D., Charters, M., Gentz, R., Parmelee, D., Li, Y., Galperina, O., Giri, J., Roschke, V., Nardelli, B., Carrell, J., Sosnovtseva, S., Greenfield, W., Ruben, S. M., Olsen, H. S., Fikes, J., and Hilbert, D. M. (1999) *Science* 285, 260–263
15. Nardelli, B., Belvedere, O., Roschke, V., Moore, P. A., Olsen, H. S., Migone, T. S., Sosnovtseva, S., Carrell, J. A., Feng, P., Giri, J. G., and Hilbert, D. M. (2001) *Blood* 97, 198–204
16. Scapini, P., Nardelli, B., Nadali, G., Calzetti, F., Pizzolo, G., Montecucco, C., and Cassatella, M. A. (2003) *J. Exp. Med.* 197, 297–302
17. Litinskiy, M. B., Nardelli, B., Hilbert, D. M., He, B., Schaffer, A., Casali, P., and Cerutti, A. (2002) *Nat. Immunol.* 3, 822–829
18. Gorelik, L., Gilbride, K., Dobles, M., Kalled, S. L., Zandman, D., and Scott, M. L. (2003) *J. Exp. Med.* 198, 937–945
19. Mackay, F., Schneider, P., Rennert, P., and Browning, J. (2003) *Annu. Rev. Immunol.* 21, 231–264
20. Khare, S. D., Sarosi, I., Xia, X. Z., McCabe, S., Miner, K., Solovyev, I., Hawkins, N., Kelley, M., Chang, D., Van, G., Ross, L., Delaney, J., Wang, L., Lacey, D., Boyle, W. J., and Hsu, H. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3370–3375
21. Kayagaki, N., Yan, M., Seshasayee, D., Wang, H., Lee, W., French, D. M., Grewal, I. S., Cochran, A. G., Gordon, N. C., Yin, J., Starovasnik, M. A., and Dixit, V. M. (2002) *Immunity* 17, 515–524
22. Sidhu, S. S., Weiss, G. A., and Wells, J. A. (2000) *J. Mol. Biol.* 296, 487–495
23. Sidhu, S. S., Lowman, H. B., Cunningham, B. C., and Wells, J. A. (2000) *Methods Enzymol.* 328, 333–363
24. Gordon, N. C., Pan, B., Hymowitz, S. G., Yin, J., Kelley, R. F., Cochran, A. G., Yan, M., Dixit, V. M., Fairbrother, W. J., and Starovasnik, M. A. (2003) *Biochemistry* 42, 5977–5983
25. Pelletier, M., Thompson, J. S., Qian, F., Bixler, S. A., Gong, D., Cachero, T., Gilbride, K., Day, E., Zafari, M., Benjamin, C., Gorelik, L., Whitty, A., Kalled, S. L., Ambrose, C., and Hsu, Y. M. (2003) *J. Biol. Chem.* 278, 33127–33133
26. Hymowitz, S. G., Filvaroff, E. H., Yin, J. P., Lee, J., Cai, L., Risser, P., Maruoka, M., Mao, W., Foster, J., Kelley, R. F., Pan, G., Gurney, A. L., de Vos, A. M., and Starovasnik, M. A. (2001) *EMBO J.* 20, 5332–5341
27. Lee, G. F., and Kelley, R. F. (1998) *J. Biol. Chem.* 273, 4149–4154
28. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* 154, 367–382
29. Weiss, G. A., Watanabe, C. K., Zhong, A., Goddard, A., and Sidhu, S. S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 8950–8954
30. Sidhu, S. S. (2001) *Biomol Eng.* 18, 57–63
31. Skelton, N. J., Koehler, M. F., Zobel, K., Wong, W. L., Yeh, S., Pisabarro, M. T., Yin, J. P., Lasky, L. A., and Sidhu, S. S. (2003) *J. Biol. Chem.* 278, 7645–7654
32. LaBean, T. H., and Kauffman, S. A. (1993) *Protein Sci.* 2, 1249–1254
33. Nilsson, B., Moks, T., Jansson, B., Abrahmsen, L., Elmblad, A., Holmgren, E., Henrichson, C., Jones, T. A., and Uhlen, M. (1987) *Protein Eng.* 1, 107–113
34. Starovasnik, M. A., O'Connell, M. P., Fairbrother, W. J., and Kelley, R. F. (1999) *Protein Sci.* 8, 1423–1431
35. Starovasnik, M. A., Skelton, N. J., O'Connell, M. P., Kelley, R. F., Reilly, D., and Fairbrother, W. J. (1996) *Biochemistry* 35, 15558–15569
36. Liu, Y., Hong, X., Kappler, J., Ling, J., Zhang, R., Xu, L., Pan, C. H., Martin, W. E., Murphy, R. C., Shu, H. B., Dai, S., and Zhang, G. (2003) *Nature* 423, 49–56
37. Chan, F. K., Chun, H. J., Zheng, L., Siegel, R. M., Bui, K. L., and Lenardo, M. J. (2000) *Science* 288, 2351–2354
38. Liu, Y., Xu, L., Opalka, N., Kappler, J., Shu, H. B., and Zhang, G. (2002) *Cell* 108, 383–394
39. Kim, H. M., Yu, K. S., Lee, M. E., Shin, D. R., Kim, Y. S., Paik, S. G., Yoo, O. J., Lee, H., and Lee, J. O. (2003) *Nat. Struct. Biol.* 10, 342–348
40. Oren, D. A., Li, Y., Volovik, Y., Morris, T. S., Dharia, C., Das, K., Galperina, O., Gentz, R., and Arnold, E. (2002) *Nat. Struct. Biol.* 9, 288–292
41. Rennert, P., Schneider, P., Cachero, T. G., Thompson, J., Trabach, L., Hertig, S., Holler, N., Qian, F., Mullen, C., Strauch, K., Browning, J. L., Ambrose, C., and Tschopp, J. (2000) *J. Exp. Med.* 192, 1677–1684
42. Kelly, K., Manes, E., Jensen, G., Nadauld, L., and Jones, D. A. (2000) *Cancer Res.* 60, 1021–1027
43. Varfolomeev, E., Kischkel, F., Martin, F., Seshasayee, D., Wang, H., Lawrence, D., Olsson, C., Tom, L., Erickson, S., French, D., Schow, P., Grewal, I. S., and Ashkenazi, A. (2004) *Mol. Cell. Biol.* 24, 997–1006